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Interaction of the *lacZ* β-Galactosidase of *Escherichia coli* with some β-D-Galactopyranoside Competitive Inhibitors

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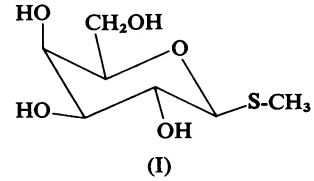
1. The location of the bivalent metal cation with respect to bound competitive inhibitors in *Escherichia coli* (*lacZ*) β-galactosidase was investigated by proton magnetic resonance. 2. Replacement of Mg^{2+} by Mn^{2+} enhances both longitudinal and transverse relaxation of the methyl groups of the β-D-galactopyranosyltrimethylammonium ion, and of methyl 1-thio-β-D-galactopyranoside; linewidths are narrowed by increasing temperature. 3. The Mn^{2+} ion is located 8–9 Å (0.8–0.9 nm) from the centroid of the trimethylammonium group and 9 Å (0.9 nm) from the average position of the methylthio protons. 4. The effective charge at the active site was probed by measurement of competitive inhibition constants (K_i^0 and K_i^+ respectively) for the isosteric ligands, β-D-galactopyranosylbenzene and the β-D-galactopyranosylpyridinium ion. 5. The ratio of inhibition constants ($Q=K_i^+/K_i^0$) obtained with 2-(β-D-galactopyranosyl)-naphthalene and the β-D-galactopyranosylisoquinolinium ion at pH 7 with Mg^{2+}-enzyme was identical, within experimental error, with that obtained with the monocyclic compounds. 6. The variation of Q for Mg^{2+}-enzyme can be described by $Q=0.1(1+[H^+]/4.17\times10^{-10})/1+[H^+]/10^{-8})$. 7. This, in the theoretical form for a single ionizable group, is ascribed to the ionization of the phenolic hydroxy group of tyrosine-501. 8. The variation of Q for Mg^{2+}-free enzyme is complex, probably because of deprotonation of the groups normally attached to Mg^{2+} as well as tyrosine-501.

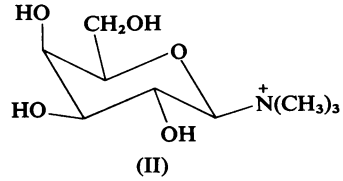
The *lacZ* β-galactosidase of *Escherichia coli* binds one Mg^{2+} cation per 125000-dalton protomer (Case *et al.*, 1973). Uptake of Mg^{2+} by apoenzyme is, kinetically, a two-step process, but no co-operativity among the four protomers is observable (Yon & Tenu, 1973). Below pH 6 there is competition between protons and Mg^{2+} (Tenu *et al.*, 1971). As is commonly the case, Mg^{2+} can be replaced by Mn^{2+}; Mn^{2+} has in fact a 10^3-fold greater affinity than Mg^{2+} for the enzyme at pH 7 (Yon & Tenu, 1973).

Removal of the bivalent metal ion does not completely inactivate the enzyme. We have advanced evidence (Sinnott *et al.*, 1978) that the Mg^{2+} is required for acidic catalysis of the departure of aglycone; we suggest that the operation of such catalysis is preceded by a conformation change which does not occur when either the Mg^{2+} is removed or the chemical structure of the substrate precludes the operation of an acidic group on the aglycone. The Mg^{2+} is not, however, directly involved as an electrophilic catalyst (Case *et al.*, 1973).

The location of the bivalent metal ion in relation to bound substrate is therefore relevant to the catalytic mechanism of this enzyme. This can be probed by n.m.r. measurements. If the bivalent metal possesses unpaired electrons, the fluctuating magnetic field from these electrons will enhance both spin–spin and spin–lattice relaxation of magnetic nuclei of bound ligands. We now report the results of studies of the methyl protons of the competitive inhibitors methyl 1-thio-β-D-galactopyranoside and the galactosyltrimethylammonium ion (I and II) binding to Mg^{2+}- and Mn^{2+}-enzyme complexes. An implicit



(I)



(II)

assumption of the method is that the binding sites for Mg^{2+} and Mn^{2+} are the same; traditionally, this is considered likely on the basis of a similarity in ionic radii (Cotton & Wilkinson, 1972). For *E. coli lacZ* β-galactosidase this traditional confidence is reinforced by the Mn^{2+}–enzyme having 82% of the activity of Mg^{2+}–enzyme against *p*-nitrophenyl galactoside (see below; also cf. Yon & Tenu, 1973).

The theory of the enhancement of proton relaxation by enzyme-bound paramagnetic ions is treated in detail by Dwek (1973). The paramagnetic contribution to the spin–lattice relaxation time (T_{1M}) and to the spin–spin relaxation time (T_{2M}) in the EI (enzyme–inhibitor) complex are given by

$$\frac{1}{T_{1M}} = \frac{2.878\times10^{-31}}{R^6}\left(\frac{3\tau_c}{1+(\omega_I\tau_c)^2}\right)$$
$$\frac{1}{T_{2M}} = \frac{1.439\times10^{-31}}{R^6}\left(4\tau_c+\frac{3\tau_c}{1+(\omega_I\tau_c)^2}\right) \quad (1)$$

where R(Å) is the distance between the nucleus under observation and the Mn^{2+} ion, τ_c is the correlation time for the interaction, and ω_I is the larmor frequency of the nucleus. This form of the Solomon–Bloembergen equations assumes the value of τ_c is such that the terms in $(\omega_S\tau_c)^2$ may be neglected and that the hyperfine terms may be neglected (Lanir & Navon, 1972). The relaxation times T_{1M} and T_{2M} may be experimentally obtained from eqn. (2):

$$\frac{1}{T_{1M}+\tau_M} = \frac{[1/T_{1(Mn^{2+})}]-[1/T_{1(Mg^{2+})}]}{f_B}$$
$$\frac{1}{T_{2M}+\tau_M} = \frac{\Delta\nu_{(Mn^{2+})}-\Delta\nu_{(Mg^{2+})}}{f_B} \quad (2)$$

where $T_{1(Mn^{2+})}$ and $T_{1(Mg^{2+})}$ are the observed spin–lattice relaxation times in solutions of ligand and either Mn^{2+}– or Mg^{2+}–enzyme, $\Delta\nu_{(Mn^{2+})}$ and $\Delta\nu_{(Mg^{2+})}$ are the observed linewidths (peak width at half height) in the same solutions, f_B is the fraction of the inhibitor in the EI complex, and τ_M is the lifetime for chemical exchange of the EI complex.

Separation of the relaxation times T_{1M} and T_{2M} from the respective measured quantities $(T_{1M}+\tau_M)$ and $(T_{2M}+\tau_M)$ is based on the expected Arrhenius-law behaviour of τ_M with temperature, that is decreasing τ_M with increasing temperature. Consequently, if $\tau_M > T_{2M}$ the lines should broaden with increasing temperature. If the lines narrow with increasing temperature then $\tau_M < T_{2M}$. Since $T_{2M} < T_{1M}$, if lines narrow with temperature one can also conclude that $T_{1M} > \tau_M$.

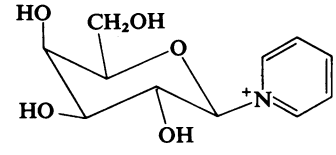
The value of τ_c can be estimated from the ratio of the paramagnetic contributions to spin–lattice and spin–spin relaxation times:

$$\frac{T_{1M}}{T_{2M}} = \frac{7+4(\omega_I\tau_c)^2}{6}$$

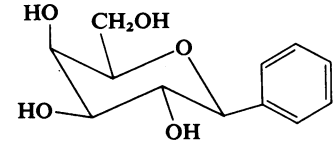
The determination of R for any given ligand proton thus requires the measurement of T_1 for solutions containing Mn^{2+}–enzyme and Mg^{2+}–enzyme, the measurement of linewidths for the same solutions, and demonstration that the broadest lines sharpen with increase in temperature.

The foregoing studies locate the bivalent metal ion with respect to aglycone protons. We have also given a preliminary account (Loeffler *et al.*, 1974) of studies in which the net effective electrostatic charge in the vicinity of the active site was probed by measurement of K_i values of isosteric inhibitors (III) and (IV), and (V) and (VI). These pairs of compounds differ only in the possession of a positive charge, and so any differences in K_i value should be caused solely by electrostatic interactions at the active site. It is useful to test this idea by also comparing the isoquinolinium (V) and 2-naphthyl (VI) compounds.

We now report studies of Q as a function of pH. The β-galactosidase protomer has 289 potentially ionizing groups (for the amino acid sequence see Fowler & Zabin, 1977). In principle ionization of any of them could, by conformation changes, influence the binding of an inhibitor (cf. Knowles, 1976). However, only those ionizations that can exert an



(III)



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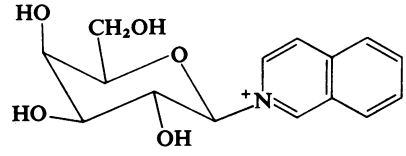

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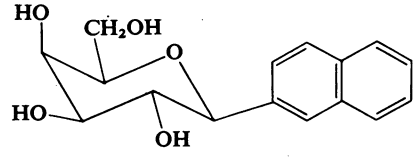

(VI)

electrostatic effect on the quaternary nitrogen of the cationic inhibitor will alter the ratio, Q, of K_i for cationic inhibitor (K_i^+) to K_i for neutral inhibitor (K_i^0). It is thus expected that only active-site ionizations will be detectable, and that conformational changes will affect isosteric inhibitors equally.

Experimental

Ligands (I)–(VI)

Our samples of the following have been described previously: methyl 1-thio-β-D-galactopyranoside (I) (Sinnott, 1971); β-D-galactopyranosyltrimethylammonium (II) bromide (Case *et al.*, 1973); β-D-galactopyranosylpyridinium (III) bromide and isoquinolinium (V) bromide (Sinnott & Withers, 1974). β-D-Galactopyranosylbenzene (IV), m.p. 152–153°C (lit. 142–143°C) (Zhdanov *et al.*, 1962), $[\alpha]_D^{25}$ +57° (*c* 0.7 in water), was made by the method used by Hurd & Bonner (1945) for the glucosyl compound. $C_{12}H_{16}O_5$ requires C, 60.0; H, 6.7 (Found C, 59.7; H, 6.7%). We found that a hygroscopic Mg^{2+}-contaminated product could be avoided only if the compound was further chromatographed on a column of Dowex-50 (H^+ form); the neutral *C*-glycoside was not retained by the ion-exchanger.

2-(β-D-Galactopyranosyl)naphthalene (VI) was made analogously. After 3 years the chromatographically homogeneous material used for K_i determination crystallized; m.p. 175–177°C, $[\alpha]_D^{25}$ +50° (*c* 1 in water). $C_{16}H_{18}O_5$ requires C, 66.2; H, 6.2 (Found C, 65.6; H, 6.9%).

lacZ β-galactosidase from *Escherichia coli* was purchased from Boehringer, Lewes, Sussex, U.K. (lot no. 7500408), Worthington, Freehold, NJ, U.S.A. (lot no. N56 359) or isolated from merodiploid strain A324-5, the kind gift of Dr. A. V. Fowler (Department of Biological Chemistry, UCLA Medical School) (Fowler, 1972).

Preparation of solutions for n.m.r. studies

Data refer to solutions 0.10M in phosphate. NaH_2PO_4,H_2O and Na_2HPO_4 were exchanged with ^2H_2O, then 0.1M solutions of each in ^2H_2O were mixed in the proportions that in H_2O would have produced a pH of 7.0. In a typical experiment, β-galactosidase (approx. 35mg) as a slurry in 2M-$(NH_4)_2SO_4$ was centrifuged, the precipitate was taken up in the deuterated phosphate buffer containing 10mM-EDTA (10ml) and concentrated at 4–5°C to approx. 1ml in an Amicon model 52 ultrafiltration apparatus, fitted with a Diaflo UM (10000-dalton exclusion) membrane. The 10mM-EDTA solution (9ml) was added, and the process was repeated. The concentration was then repeated 3 times with the deuterated sodium phosphate buffer containing only 0.25mM-EDTA. The final enzyme solution was centrifuged (approx. 10000*g* for 20min). Active-site concentrations were calculated on the assumption that $k_{cat.}$ for *p*-nitrophenyl β-D-galactopyranoside at pH7.0 and 25°C, hydrolysed by Mg^{2+}–enzyme, was $156 s^{-1}$ (cf. Sinnott & Souchard, 1973). This is some 20% higher than the value corresponding to the specific activity obtained by Tenu *et al.* (1971). An error of a factor of 2 in the concentration of active sites introduces a 10% error in distance estimates.

To this enzyme solution (0.30ml) in an n.m.r. tube was added either a 0.279M solution of inhibitor (II) bromide (30μl), or a 0.36M solution of inhibitor (I) (30μl), and a solution of 30mM-$MnCl_2$ (4.5μl) or 30mM-$MgCl_2$ (4.5μl), all substances being in solution in 99.7% ^2H_2O.

N.m.r. measurements

Spectra were recorded on a JEOL JA-100 spectrometer in the Fourier-transform mode locked on HO^2H. T_1 data were obtained by using a standard 180°–τ–90° pulse sequence. T_1 values obtained from the program package of this machine were checked by calculating first-order rate constants from peak heights manually. Some data on inhibitor (II) were obtained on a Bruker HXS-270 n.m.r. spectrometer.

Measurements of K_i as a function of pH

At pH7.0, 72 measurements of the initial rate of hydrolysis of *p*-nitrophenyl galactoside by apoenzyme and Mg^{2+}–enzyme, at each of nine concentrations of inhibitors (III)–(VI) and eight substrate concentrations, were made. At other pH values, and with Mg^{2+}-free enzyme, measurements were made at only one substrate concentration, and K_i values were calculated from

$$\frac{v_0}{v} = 1 + \frac{[I]}{K_i} \cdot \frac{K_m}{[S]+K_m}$$

Concentrations of [S] and K_m were chosen. At pH7 with both apo- and holo-enzyme this procedure gave the K_i values within 10% of those obtained from 64 measurements of initial rate (at eight substrate concentrations, ranging from $7K_m$ to $K_m/7$, and eight inhibitor concentrations).

Results and Discussion

Location of bivalent metal cation

Fig. 1 shows the methyl proton resonance of methyl thiogalactoside in the presence of Mg^{2+}– and Mn^{2+}–enzyme. There is significant, but not huge, broadening for the Mn^{2+}–enzyme, indicating immediately that the Mn^{2+}, although in the proximity of the active site, is not co-ordinated to the sugar. Enzyme from which the Mg^{2+} had been removed had 82% of the activity towards *p*-nitrophenyl galactoside (10mM) in 0.1M-sodium phosphate buffer (pH7.0)/0.5mM-EDTA/

1mM-$MnCl_2$ as it did towards the same substrate solution but containing 1mM-$MgCl_2$, whereas complete removal of Mg^{2+} causes a 6-fold decrease in $k_{cat.}$ (Sinnott *et al.*, 1978); it is therefore likely that Mg^{2+} and Mn^{2+} are occupying the same sites.

Similar qualitative observations were made for inhibitor (II), confirming our findings (Case *et al.*, 1973) that the bivalent metal remains bound even in the presence of a cationic inhibitor. Qualitatively also the resonances attributable to the protons at C-6 and C-5 of both inhibitors are broadened comparably with the methyl resonances, indicating the

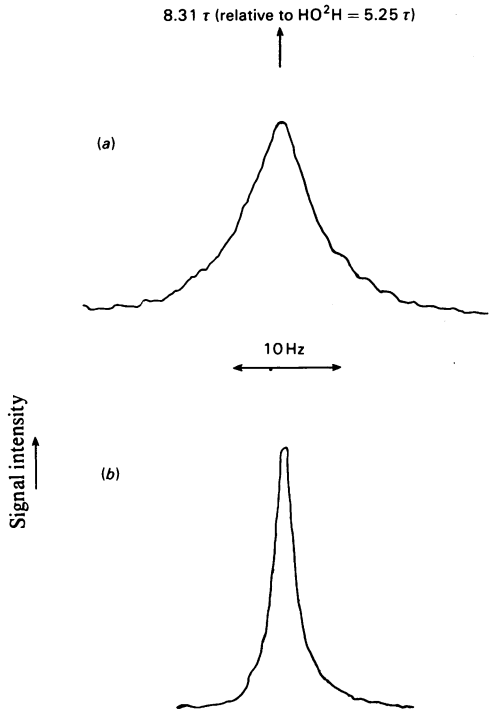

Fig. 1. *Methyl peak in the p.m.r. spectrum of methyl 1-thio-β-D-galactopyranoside in the presence of (a) 154μM-Mn^{2+}–β-galactosidase and (b) 154μM-Mg^{2+}–β-galactosidase*
Field strength increases from left to right.

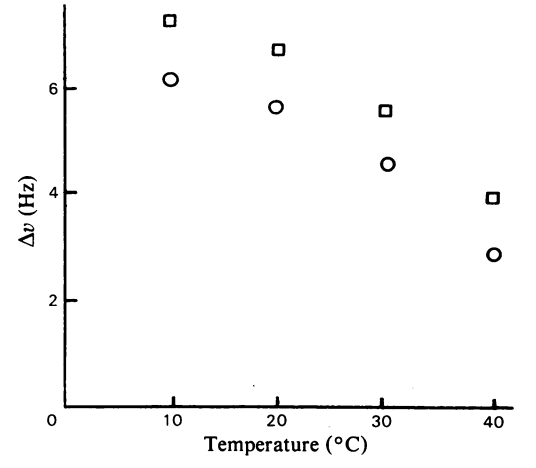

Fig. 2. *Width at half-height of the methyl peak in the p.m.r. spectrum of neutral inhibitor (I) (○) (17mM) and cationic inhibitor (II) (□) (13mM) in the presence of 80μM-Mn^{2+}-β-galactosidase, as a function of temperature*
The narrowing of lines with increase in temperature indicates that exchange between free and bound ligand is fast in both cases.

Table 1. *P.m.r. relaxation data at 27±3°C*
For meanings of symbols see the introduction. $[X]_0$ denotes the total concentration of a substance X.

Aglycone	$[E]_0$ (μM)	$[I]_0$ (mM)	$10^3 f_B$	$[Mn^{2+}]_0$ (mM)	$[Mg^{2+}]_0$ (mM)	$[EDTA]_0$ (mM)	$10^8\omega_I$ (s^{-1})	T_1 (s)	Δν (Hz)	$10^9\tau_{cc}$ (s)	R/Å
SMe	84	32.7	2.4*	0.40	0	0.25	6.28	0.36	7.4		
	84	32.7	2.4	0	0.40	0.25	6.28	1.44	2.3	7	~9
	0	32.7	0	0.41	0	0.25	6.28	0.57	1.9		
	0	32.7	0	0.41	0	0.335	6.28	0.58	1.7		
	0	30	0	0	0	1.0	17.0	1.30	1.4		
$-NMe_2^+$	84	25.4	3.1†	0.40	0	0.25	6.28	0.14	12.9		
	84	25.4	3.1	0	0.40	0.25	6.28	0.78	3.8	5	8
	0	25.4	0	0.41	0	0.25	6.28	0.23	2.9		
	0	25.4	0	0.41	0	0.335	6.28	0.22	2.9		
	55	14	3.5	0.67	0	0.50	17.0	0.17	11.1		
	55	4	9.5	0.67	0	0.50	17.0	—	20.0		
	55	14	3.5	0	0.67	0.50	17.0	0.53	6.1	2	9
	0	14	0	0.67	0	0.50	17.0	0.21	4.0		
	0	16	0	0	0	1.00	17.0	0.50	1.9		

* Assuming K_I=2mM (Sinnott, 1971).
† Assuming K_I=1.8mM for all enzyme species (Case *et al.*, 1973).

metal ion is towards rather than away from the glycone.

The linewidths of the methyl resonances of both inhibitors binding to Mn^{2+}–enzyme are given in Fig. 2 as a function of temperature; lines narrow with increasing temperature. Therefore fast exchange conditions obtain, $T_{2M} > \tau_M$, and the quantitative treatment outlined in the introduction is applicable. Pertinent data are given in Table 1.

These data confirm that most of observed line broadening in the presence of Mn^{2+}–enzyme is indeed caused by a specific interaction with Mn^{2+}–enzyme. However, the T_1 value in the presence of Mn^{2+} alone approaches that in the presence of Mn^{2+}–enzyme. This relaxation enhancement is not a function of free Mn^{2+} concentration since it is unchanged in the presence of additional EDTA, but rather a consequence of having Mn^{II} distributed uniformly throughout the solution. We correct for it by writing

$$\frac{1}{T_{1M}} = \frac{[1/T_{1(Mn^{2+}\text{–enzyme})}] - [1/T_{1(Mg^{2+}\text{–enzyme})}] - \{[1/T_{1(Mn^{2+}\text{ alone}} - 1/T_{1(0)}]\}}{f_B}$$

and likewise

$$\frac{1}{T_{2M}} = \frac{\pi\{\Delta\nu_{(Mn^{2+}\text{–enzyme})} - \Delta\nu_{(Mg^{2+}\text{–enzyme})} - [\Delta\nu_{(Mn^{2+}\text{ alone})} - \Delta\nu_{(0)}]\}}{f_B}$$

where $T_{1(0)}$ and $\Delta\nu_{(0)}$ refer to the inhibitor in the absence of metal or enzyme.

The resulting values of R are approximate (although the latter correction is small), but such is the insensitivity of this method to the precise parameters used in the calculation that correction for non-enzyme Mn^{2+} increased R by only 1Å (1.1nm) in ion (II) and 2Å (0.2nm) in compound (I); we therefore estimate the error in this distance measurement to be about ±1Å (0.1nm).

The bivalent metal ion in β-galactosidase is thus about 8–9Å (0.8–0.9nm) from the average position of the methyl protons of galactosyltrimethylammonium bromide and very approximately 9Å (0.9nm) from the methyl protons of methyl thiogalactoside. However, because of the R^6 dependence of relaxation effects on distance, the 'average' position of these methyl protons is not simply ascertainable. For a methyl group simply rotating (Rowan *et al.*, 1974) the centre of the CH_3 triangle can be taken as the proton position if the CH_3 group is rotating fast. Such must be the case in our system, since slow methyl (or NMe_3) rotation would constitute a form of chemical exchange; the narrowing of the lines on increasing the temperature has shown that all such processes are fast compared with paramagnetic relaxation. For inhibitor (II) the average position of the nine equivalent protons is presumably approximated at large distances by the centre of the triangle defined by the centroids of the individual CH_3 groups.

For inhibitor (I), the average position of the methyl protons is impossible to define accurately, since rotation can occur both about the C-1 and about the S–CH_3 bonds; the distance measurements are in any event less precise.

These experiments do not locate the Mn^{2+} more accurately than about ±1Å (0.1nm) with respect to the active site, but do show clearly that the bivalent metal is too far away to be directly involved in catalysis, but near enough to modulate, for example, a conformational change. Kinetic evidence (Sinnott *et al.*, 1978) indicates that the bivalent metal is necessary for the operation of acid catalysis in the departure of aglycone. Affinity-labelling studies (Sinnott & Smith, 1978) have demonstrated the presence in the active site of methionine-500; residue 501 is tyrosine. The conformational change in the hydrolysis of aryl galactosides in the presence of Mg^{2+} that we proposed on the basis of kinetic arguments (Sinnott & Souchard, 1973) has now been detected by low-temperature studies (Fink, 1977) and described as 'substantial'. This must involve the large perturbation of an enzyme chromophore.

The precedent of carboxypeptidase makes the hypothesis that the conformational change in β-galactosidase action is also the motion of a tyrosine side chain attractive. The acid catalyst would then be the phenolic hydroxy group.

Net charge at the active site

Table 2 gives values of K_i for inhibitors (III)–(VI) under various conditions. The pyridinium salts (III) and (V) are in fact substrates, so the competitive inhibition constants are K_m values. However, $k_{cat.}$ for these ligands is small ($\sim 1\,s^{-1}$) and this value represents the rate of the first step after formation of the Michaelis complex (Sinnott & Withers, 1974). Therefore $K_i = K_m = K_s$. It is intuitively expected that quaternary pyridinium salts are isosteric with alkylbenzenes, and there are X-ray-crystallographic data to confirm this. The molecular dimensions of the aromatic rings of 4,4′-dimethylbiphenyl (Casalone *et al.*, 1969) and the *NN*′-dimethylbipyridylium ion (Russell & Wallwork, 1972) are identical, within experimental error, with the exception of a C–CH_3 bond longer (0.05Å; 0.005nm) than a N–CH_3 bond. Even this difference is far less than the amplitude of

thermal vibrations in the crystal, and indeed an apparent bond-length difference of 0.03Å (0.003 nm) has been considered to arise from inadequate correction for these motions (Bottrill *et al.*, 1975).

Nonetheless, we considered it worthwhile to test further the validity of this idea by using the isoquinolinium–2-naphthyl system as well as the pyridine–benzene system. The data for inhibitors (V) and (VI) give, within experimental error, the same ratio (Q) of K_i values ($Q=K_i^+/K_i^0$) at pH 7.0 as the monocyclic pair of inhibitors; therefore the small changes in bond length and bond angle between pyridinium and benzene derivatives do not alter values of Q. In Figs. 3 and 4 $\log Q$ is plotted as a function of pH: the logarithmic presentation ensures roughly constant error bars. Our expectation that Q, unlike almost any other steady-state kinetic parameter, would be susceptible to simple interpretation is fulfilled for the Mg^{2+}–enzyme.

Table 2. *Binding constants of ligands (I) and (II) to apo- and holo-enzyme*

Values are mM, errors being the standard deviations upon the gradients of the linear plots from which the K_i values were derived.

	Mg^{2+}		No Mg^{2+}	
pH	K_i^+	K_i^0	K_i^+	K_i^0
5.0	7.28±1.00	5.06±0.37	17.02±0.61	19.59±0.83
5.5	4.28±0.25	2.30±0.10	6.26±0.70	5.74±0.20
6.0	3.81±0.04	1.63±0.16	2.18±0.07	2.32±0.27
6.5	2.42±0.05	1.16±0.10	2.13±0.03	4.58±0.11
7.0	1.12±0.02	0.45±0.03	2.88±0.32	6.74±0.37
7.5	1.80±0.08	0.99±0.02	3.00±0.07	26.0 ±4.0
8.0	2.30±0.11	1.90±0.09	5.30±0.21	44 ±3
8.5	2.77±0.12	4.47±0.09	10.03±0.54	68 ±7
9.0	1.99±0.02	5.85±0.05	4.55±0.27	>100
9.5	2.92±0.10	9.66±0.36	2.09±0.10	
7.0*	0.91	0.39		

* Compounds (V) and (VI).

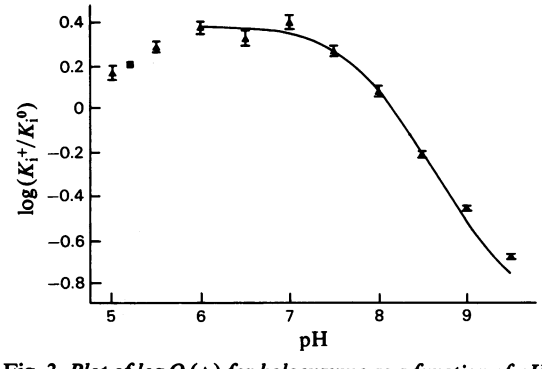

Fig. 3. *Plot of log Q (▲) for holoenzyme as a function of pH*

For conditions see the text. ■, Q corrected for competition between protons and Mg^{2+}. The continuous line is calculated from

$$Q=\frac{0.1(1+[H^+]/4.17\times 10^{+10})}{(1+[H^+]/10^{-8})}$$

Error bars are derived from the errors given in Table 2.

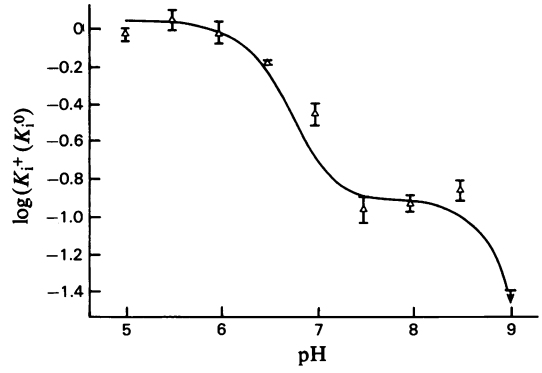

Fig. 4. *Plot of log Q for apoenzyme as a function of pH*

For conditions see the text. The arrow denotes a maximum value of Q. Error bars are derived from the errors given in Table 2.

Q for holoenzyme. The low-pH fall in Q is entirely attributable to the previously noted (Tenu *et al.*, 1971) competition between protons and Mg^{2+}. The two lowest-pH points pertain to solutions containing 5.0 mM-$MgCl_2$, but this is still insufficient to saturate the enzyme with Mg^{2+}. A binding constant of 7 mM can be calculated for Mg^{2+} at pH 5.16 (Tenu *et al.*, 1971). In Fig. 3 is shown the value of Q calculated from this value, a true value of Q of 2.4 for Mg^{2+}–enzyme, and the value of Q of 1.0 for Mg^{2+}-free enzyme at high pH.

The change in Q at low pH can be accounted for by a single ionization. To what species this ionization refers is not intuitively obvious. $\log Q$ measures the difference in Gibbs free energy between the EI complexes with neutral and with cationic inhibitors; free enzyme is not involved. Therefore changes in Q with pH will reflect ionization of EI complexes. In fact, for a single ionization of these complexes, Q is given by:

$$Q=Q_0\frac{\left(1+\frac{[H^+]}{K_a^0}\right)}{\left(1+\frac{[H^+]}{K_a^+}\right)} \qquad (5)$$

where Q_0 is the limiting value of Q at high pH, K_a^+ is the acid dissociation constant of the EI com-

plex with cationic inhibitor and K_a^0 is the analogous quantity with neutral inhibitor.

The data in Fig. 3 fit this expression with $Q_0=0.1$, $pK_a^+=8.0$ and $pK_a^0=9.38$. Because the equilibria are linked, the value of Q at low pH is given by $Q_0(K_a^+/K_a^0)$, in this case 2.4.

Since the inhibitors are isosteric, the perturbation in the pK_a of the ionization on binding a cationic inhibitor (1.38 units) must be attributable entirely to electrostatic interactions; the effects of conformational changes will be the same for the two inhibitors. This perturbation of the pK_a in the EI complex can also be expressed as a 24-fold decrease in Q on deprotonation of the entire system. This purely electrostatic effect is large, and the ionizing group must consequently be in the active site, in the vicinity of the positive charge. A rough estimate of the distance involved can be obtained by equating the change in free energy of binding, consequent on deprotonation, with the electrostatic potential energy of two point charges rÅ apart. This gives $r=176/\varepsilon$, where ε is the 'effective dielectric constant'. Reasonable values of ε give r a value between 2 and 9Å (0.2 and 0.9nm).

We have affinity-labelled β-galactosidase with β-D-galactopyranosylmethyl-*p*-nitrophenyltriazene; in the active site of the enzyme this compound is decomposed to give the β-D-galactopyranosylmethyl cation, which is captured with 80% efficiency by the sulphur atom of methionine-500 of Mg^{2+}–enzyme to give a sulphonium salt (Sinnott & Smith, 1978). It is therefore a reasonable assumption that the sulphur of methionine-500 is close to the cationic centre.

This cationic centre is in exactly the same place, relative to the glycone, as the positive charge of the pyridinium salt. Examination of Dreiding models of the dipeptide Met-Tyr (cf. residues 500 and 501) indicates that the maximum distance possible between the methionine sulphur and the phenolic hydroxy group is ~12Å (1.2nm), but this can be achieved only with an extended conformation. Therefore the maximum distance between the phenolic hydroxy group and the positive charge of the pyridinium salt is 13–14Å (1.3–1.4nm), but this can only be achieved by interposing largely hydrocarbon residues between the two charges. This would result in a low value of ε. Any arrangement whereby the space between positive charge and the phenolic hydroxy group would be occupied by water would require the groups to be much closer together. Therefore, unless the protein conformations in the complexes with triazene, galactosylpyridinium salt and galactosylbenzene are very different, the ionization of tyrosine-501 must be reflected in a change in the value of Q.

The simplest proposal, therefore, is that the ionization observed is that of tyrosine-501. In the EI complex with neutral inhibitor the pK_a is very similar to that of a simple phenol (10; Barlin & Perrin, 1966).

Q for apoenzyme (*Fig.* 4). The value of Q for Mg^{2+}-free enzyme is not so susceptible of simple interpretation: the data are in any event of lower accuracy than those for Mg^{2+}–enzyme, since K_i^0 values become high above pH7.0. Nonetheless some conclusions are warranted.

The limiting value of Q at low pH (1.0) is less than that for Mg^{2+}–enzyme, but this fall in the low-pH value of Q on removal of Mg^{2+} is too small for it to have been caused by the simple removal of the two electronic charges of the Mg^{2+}. Such an interpretation requires the cation to be a distance $1277/\varepsilon$Å away from the pyridine nitrogen. Even if the dielectric constant of pure water is used, this still gives a distance of 16Å (1.6nm), considerably greater than the distance from the centroid of the trimethylamino group of compound (II) to bound Mn^{2+}, estimated from n.m.r. measurements. In fact the competition between Mg^{2+} and protons (Tenu *et al.*, 1971) makes it probable that the Mg^{2+} is being replaced by protons. The increase in the dissociation constant for Mg^{2+} binding from 1μM at pH7 to 7mM at pH5.2 makes it likely that two of the co-ordinating groups of Mg^{2+} are being protonated. The fall in Q at low pH on removal of Mg^{2+} then merely means that the co-ordinating groups are further away from the active site than the Mg^{2+} itself.

These groups, however, will probably deprotonate in the pH range studied. Therefore, in addition to tyrosine-501, the groups that in holoenzyme co-ordinate Mg^{2+} will also deprotonate. Q for Mg^{2+}-free enzyme will then be governed by six pK_a values. The data cannot be analysed on this model, although the generally complex variation is expected.

General Discussion

If the conformation of the protein in the two EI complexes is the same, the ratio of the binding constants of isosteric quaternary ammonium and quaternary carbon compounds is a measure of the net charge at an enzyme active site. Contributions to this net charge arise both from ionized groups on the protein and from any bound metals.

For the *lacZ* β-galactosidase of *Escherichia coli*, one possible contributor to the net charge is the bound bivalent metal ion. This metal ion was located independently by magnetic-resonance methods, and found to be 8–9Å (0.8–0.9nm) from the aglycone of a competitive inhibitor bound in the active site. At such a distance it cannot play a catalytic role, but it is close enough to modulate the motion of an acid-catalytic group.

Removal of Mg^{2+}, however, does not have a marked electrostatic effect, far less than one would expect from removal of two full positive charges from

only 8–9Å (0.8–0.9nm) away. However, it is known that there is strong competition between Mg^{2+} and protons (Tenu *et al.*, 1971) in binding to the protein. A hypothesis that accords with the data of Tenu *et al.* (1971) and our 'electrostatic' and n.m.r. measurements is that when the Mg^{2+} is bound to apoenzyme the co-ordinating groups are deprotonated.

The ratio of the binding constants of the differently charged inhibitors will vary with pH as groups near the active site are deprotonated. However, for apoenzyme, at the very least the two groups that normally bind Mg^{2+} will deprotonate in the accessible pH region, and the variation is too complex to be analysed with data that are practicable to obtain. For holoenzyme, however, the data can be analysed in terms of a single ionization.

The magnitude of the change in the ratio of binding constants for the isosteric inhibitors as the active-site group is deprotonated can give, from simple electrostatic considerations, some indication of the distance of the ionizing group from the positive charge. From the results of affinity labelling with a reagent that generates an electrophilic centre at precisely the site occupied by the quaternary nitrogen of ligand (III), it is possible to deduce that deprotonation of the phenolic hydroxy group of tyrosine-501 must produce an electrostatic effect on binding of ligands (III) and (IV).

The role of tyrosine-501 could be further probed by substituting fluorotyrosine for tyrosine in the β-galactosidase synthesized by a suitable strain of the bacterium; modification of pre-existing protein by such reagents as tetranitromethane is unlikely to give clear results on an enzyme of this size. With the determination of the primary sequence of this enzyme (Fowler & Zabin, 1977), X-ray-crystallographic studies of the tertiary structure are now possible. Such work may be expected to provide a clear test of these proposals about the disposition of the active-site residues of β-galactosidase.

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